REMARKS

For purposes of the remarks that follow, Applicant assumes that his prior response (filed October 24, 2003) on the merits has been entered. Thus, the claim set submitted herewith is for the Examiner's convenience. The claims remain unchanged from Applicant's prior response.

Applicant explicitly reiterates and incorporates herein the comments made in his prior response. The additional remarks that follow address the issues presented in the Office Action dated August 12, 2004.

The Recitation of WO 98/13694:

In the prior response, Applicant amended the specification at page 1, in the paragraph spanning lines 7-19 to delete a reference to "WO 97/02667" and to insert in its place a reference to WO 98/13694. This was done to correct an obvious typographical error. Applicant apologizes for not explicitly pointing this out in his prior response.

Specifically, the application number of Applicant's earlier application is not "**WO** 97/02667," but rather "**PCT/GB97**/02667." In other words, in the application as filed the actual identifying number was correct, but the prefix was not. The correct WO publication number for PCT/GB97/02667 is "**WO 98/13694**," the number Applicant inserted into the application in the prior response.

A complete copy of WO 98/13694 is attached hereto as Exhibit A. Note that the priority document "PCT/GB97/02667" is referenced on the cover sheet. Applicant thus submitted the earlier change to page 1 of the specification to correct a typographical error.

New Matter and Incorporation by Reference:

Applicant respectfully traverses the Office's indication that the prior response inserts new matter. The Office, however, did not identify the allegedly new matter with specificity. Applicant assumes that issue involves, at least in part, the WO publication numbers discussed hereinabove. On this point, Applicant submits that the documents incorporated by reference support the matter previously added. Also, in response to the

Examiner's request for a more formal incorporation by reference, the specification has been amended herein to include the critical data presented in the earlier applications.

Applicant respectfully submits that the polypeptide, ISRFAWGEV, reference to which was inserted into the present application in Applicant's prior response, is explicitly referenced in both WO 98/13694, attached hereto as Exhibit A, and in WO 99/47932, attached hereto as Exhibit B and explicitly referenced in the application as filed at page 1, line 21. (Both of these references were included on the International Search Report in this case and are thus already party of the official file.) Thus, Applicant respectfully submits that no new matter has been inserted into the specification or claims.

Regarding a line-by-line indication of support for the present claims, the following table is an element-by-element concordance between the language of the present claims and the corresponding basis for support in the application as filed and amended. Please note that the page and line numbers refer to the corresponding PCT Application as filed.

Support for the Claims as Amended in Response Dated October 24, 2003

Claim	Subject Matter	Support
11	antibodies that bind to Acinetobacter species	Claim 7 as filed
	that present to the vertebrate an antigen that mimics the myelin of the vertebrate	p. 1, lines 13-16
	in which the Acinetobacter species contains the sequence ISRFAWGEV	See table on p. 2 of WO 98/13694 (*i.e.*., PCT/GB97/02667) (attached hereto as Exhibit A)
12	at least two of	p. 2, lines 9-11 p. 5, lines 3-8
13	in which the antibodies are IgA antibodies	p. 2, line 9
14	in which the antigens used for assaying antibodies to neurofilaments are	Claim 9 as filed p. 2, lines 26-30
15	which includes the assay of antibodies (c)	p. 5, lines 3-4
16	in which the test antigen for antibodies (c) is whole Acinetobacter	p. 3, 2nd ¶ of WO 98/13694
17	in which the test antigen for antibodies (c) is a peptide having a sequence that mimics the myelin of the vertebrate	p. 2, last ¶ of WO 98/13694 p. 3, 2nd ¶ of WO 98/13694
18	in which the test antigen for antibodies (c) is a peptide having the sequence ISRFAWGEV	Table on p. 2 of WO 98/13694

19	in which the determined levels of antibodies are combined and compared with those present in control samples	p. 5, lines 11-19
20	in which the multiplication product of the determined levels of the antibodies assayed is calculated	p. 5, lines 11-16
21	in which the multiplication product of all three antibodies is calculated	p. 5, lines 11-16
22	in which a positive result is indicated by a multiplication product of at least three standard deviations above that of control samples	p. 5, lines 28-29
23	in which the Acintobacter species is *Acinetobacter calcoaceticus*	p. 5, line 8
24	an antigen specific for antibodies to an Acinetobacter species containing a peptide sequence that mimics the myelin of the vertebrate	Claim 7 as filed p. 1, lines 13-16
	at least two of	p. 2, lines 9-11 p. 5, lines 3-8
25	in which the Acintobacter species contains the sequence ISRFAWGEV	Table Table on p. 2 of WO 98/13694
26	in which the Acintobacter species is *Acinetobacter calcoaceticus*	p. 5, line 8
27	in which the test antigen for antibodies (c) is whole Acinetobacter	p. 3, 2nd ¶ of WO 98/13694
28	in which the test antigen for antibodies (c) is a peptide having a sequence that mimics the myelin of the vertebrate	p. 2, last ¶ of WO 98/13694 p. 3, 2nd ¶ of WO 98/13694
29	in which the test antigen for antibodies (c) is a peptide having the sequence ISRFAWGEV	Table on p. 2 of WO 98/13694
30	in which all three test antigens are present	p. 5, lines 3-8
31	in which the antigens used for assaying antibodies to neurofilaments are	Claim 9 as filed p. 2, lines 26-30
32	for use in an assay for IgA antibodies	p. 2, line 9
33	kit comprising as test antigens: myelin, neurofilaments, and an antigen specific for an antibody to an Acinetobacter species containing a peptide comprising ISRFAWGEV	Claim 8 as filed Table on p. 2 of WO 98/13694
34	in which the test antigen for antibodies (c) is a peptide having the sequence ISRFAWGEV	Table on p. 2 of WO 98/13694
35	assaying a biological sample for IgA antibodies which bind to myelin and/or neurofilaments, or to one or more antigenic (immunogenic) parts thereof	Claim 2 as filed
36	in which a positive result is indicated by levels of antibodies at least about two standard deviations above that of control samples	Claim 6 as filed

Applicant thus submits that the application as filed, in combination with the matter which was originally incorporated by reference, fully supports the scope of hte present claims. In accordance with the Examiner's recommendation, the essential matter originally incorporated by reference has been inserted into the specification.

Election by Original Presentation:

The Office has taken the position that Claims 12, 14, 18, 25, 29, 31, 33, and 34 are independent or distinct from the invention originally submitted by the Applicant "due to new matter." Because, as noted above, no new matter has been introduced to the specification or claims, this position is respectfully traversed. Both the invention as originally presented and as amended is drawn to a method for diagnosing spongiform disease or demyelinating disease in vertebrates. Applicant has not, so to speak, "switched gears" in mid-prosecution. Nor has Applicant added any new matter to the application. Applicant is entitled to define the invention as best he can. So long as that definition is clear, the Office has a duty to examine Applicant's claims on their merits.

Additionally, and for purposes of compact prosecution, Applicant further traverses this rejection because the Office has not supported its holding (which amounts to a restriction requirement) by way of reasons or examples, as required by MPEP §803. In the absence of any sound rationale or examples, it is insufficient for the Office simply to conclude that the claims as amended define "an independent or distinct" invention.

The Prior Response in PCT/GB/03936:

The Office indicates that Applicant's prior submission of a response in PCT/GB/03936 is acknowledged, but was not considered on the merits because the prior submission was not signed. Attached hereto as Exhibit C is a signed copy of the earlier response. Applicant respectfully requests that Exhibit C be entered and considered on it merits.

Applicant's Prior Remarks and the Rejection of Prior Claims 1, 3, 4, and 7 Under 35 USC §102(b) in View of Ebringer et al. (11/1997) Environmental Health Perspectives 105(11):1172-1174 or Ebringer WO 98/13694):

Applicant respectfully submits that his prior remarks with regard to this rejection were neither vague, nor circular, nor confusing. Rather, Applicant respectfully submits that the opening remark in his prior response was a model of perfect clarity: "This rejection has been rendered moot by cancellation of the claims." In short, in the Action dated June 30, 2003, the Office rejected Applicant's prior Claims 1, 3, 4, and 7. Claims 2 and 6 were indicated as containing allowable subject matter.

In response, all of original Claims 1-10 were canceled. The claims containing allowable subject matter were reformulated as independent Claims 35 and 36. The remaining subject matter was then recast in a new set of claims. Insofar as the explicit rejection of Claims 1, 3, 4, and 7 had been rendered moot by canceling the rejected claims, Applicant then went on to offer his comments on the potential applicability of the cited references to the newly submitted claims.

Specifically, Applicant noted that the §102 rejection in view of Ebringer et al. (11/1997) *Environmental Health Perspectives* 105(11):1172-1174 or Ebringer WO 98/13694) are be inapplicable to the new claims because Claim 11 is limited to a method of assaying **for at least two of the three listed types of antibodies**. This aspect of the present invention is not disclosed by either of the applied references (or both of them taken together) and thus a rejection based on anticipation is improper.

The Applicant went on to note that the Office appeared to be making an unwarranted assumption in applying this combination of references in the first instance. As noted in Applicant's prior response:

> The Office assumes that assaying an antibody which binds to Acinetobacter also assays antibodies which bind myelin or an antigenic peptide that exhibits molecular mimicry of a mammalian myelin peptide, due to cross-reactivity. This assumption, however, is incorrect. As emphasized in the Response to the Written Opinion filed during the international phase (signed copy enclosed), the present invention relies on **separate** measurements of antibodies to myelin, neurofilaments, and Acinetobacter (please see page 5, line 6 of the application as filed).

Moreover, as stated above, assaying more than one type of antibody greatly improves the specificity of the diagnostic method over the assaying for antibodies to Acinetobacter alone (as disclosed in the cited documents). Neither of the cited documents suggests or hints at the possibility of a diagnostic test using the materials positively recited in Claim 11. It was not within the foresight of even the present inventor to propose the claimed method. This being despite the fact that he is the champion of the theory of a molecular mimicry mechanism underlying these diseases.

In short, the two Ebringer references neither disclose nor suggest the invention positively recited in Claim 11. Applicant therefore submits that Claim 11 and the claims dependent thereon are both novel and non-obvious in view of the prior art now of record.

For the reasons given above, independent kit claim 14 (previous claim 8) has also been limited such that the kit comprises, as test antigens at least two of myelin, neurofilaments and an antigen specific for antibodies to Acinetobacter.

CONCLUSION

Applicants submit that the application is now in condition for allowance. Early notification of such action is earnestly solicited.

Respectfully submitted,

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(54) Title: DIAGNOSIS OF SPONGIFORM DISEASE

(57) Abstract

A diagnostic test is provided for spongiform encephalopathy and other demyelinating conditions in mammals which comprises assaying antibodies present in the mammal which bind to an antigenic peptide which exhibits molecular mimicry of a mammalian myelin peptide, e.g. one having the sequence FSWGAEGQK. This test is useful for detecting BSE in cattle by assaying sera collected from the cattle for antibodies to a species of Acinetobacter, Agrobacterium or Ruminococcus, or a peptide having a sequence present in said species which mimics a peptide of bovine myelin and identifying animals having a level of antibodies at least about two standard deviations above that of healthy control animals.

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DIAGNOSIS OF SPONGIFORM DISEASE

This invention relates to the detection of spongiform encephalopathy and other demyelinating conditions in mammals and is particularly, but not exclusively, concerned with the diagnosis of bovine spongiform encephalopathy (BSE).

BSE is a recent neurological disorder of cattle, which was first reported in the U.K. after 1982, following a change in the preparation of "bone and meal" feeds. BSE has attracted some public concern, lest it be transmitted to humans following meat consumption. It has been suggested that BSE is caused by "prions", a type of infectious protein.

The present invention is based on an alternative model of the genesis of various forms of spongiform encephalopathy and other demyelinating conditions in mammals. According to the proposed model, BSE and related diseases are conceived as autoimmune diseases arising as a result of molecular mimicry between certain infective agents and the myelin of the infected mammal. This new model of BSE, in particular, is based on the following experimental observations.

A characteristic histopathological feature of BSE is a "spongiform" appearance, which also occurs in chronic but not acute "experimental allergic encephalomyelitis" (EAE), at least in rabbits and guinea pigs. A short sequence of bovine myelin (FSWGAEGQK), which withstands denaturation following heating to 100°C for one hour, was reported over twenty-five years ago to produce hind quarters paralysis, tremors and death, following inoculation into guinea pigs, which to some extent resembles the features observed in cattle suffering from BSE. In accordance with the present invention, this sequence has been used as a computer probe to search for proteins showing molecular mimicry. This sequence, in denatured form, may be described as encephalitogenic.

Analysis of proteins in databases (Genbank and SwissProt) revealed that 3 microbes showed molecular mimicry of the bovine myelin sequence , the best one being found in 4-carboxy-muconolactone-decarboxylase of Acinetobacter calcoaceticus, a common microbe present in soil and water supplies. These sequence similarities are shown in the following Table.

Comparison of amino acids of bovine myelin to microorganisms from Genbank and SwissProt which have similar sequences in other proteins.

Source	Amino acids	Positions	Locations
Bovine myelin	LSRFSWGAE	110 - 118	
Acinetobacter calcoaceticus	ISRFAWGEV	41 - 49	4-carboxy-muconolactone decarboxylase
Agrobacter tumefaciens	YTRFTWGAP	693 - 701	Beta-glucosidase
Ruminococcus albus	YTQFEISAE	274 - 282	Beta-glucosidase

Alphabetic letters refer to biochemical symbols for amino acids.

In conformity with the new model, it has now been found that sera of BSE affected cattle contain significantly high levels of antibodies to Acinetobacter species.

The present invention therefore provides a diagnostic test for spongiform encephalopathy and other demyelinating conditions in mammals which comprises assaying antibodies present in the mammal which bind to an antigenic peptide which exhibits molecular mimicry of a mammalian myelin peptide, especially one having the sequence FSWGAEGQK. The term "molecular mimicry" refers to a degree of similarity (sequence homology) as between the antigenic peptide and a myelin peptide which results in the formation of antibodies which cross-react with myelin and demyelinate nervous tissue. The presence of such antibodies at elevated levels compared to

those found in unaffected animals is therefore a marker for BSE which may be used to detect BSE at an early stage at which curative or other appropriate action may be taken.

The assay may be carried out using the whole Acinetobacter or other organism as the test antigen. Any strain of Acinetobacter having the antigenic peptide identified above may be used. Alternatively the isolated peptide or a synthetic form of the peptide may be used as antigen. Any suitable type of assay procedure may be used, the ELISA method being especially convenient.

Antibody levels indicative of BSE are those which are significantly higher than the control levels. Usually, levels elevated to about 2 standard deviations above the controls may be taken as a positive indication but margins around this figure may be possible or desirable for purposes of caution.

Procedures for carrying out an assay in accordance with this invention are described in the following illustrative Example, based on comparison of sera from animals known to have had BSE with sera from healthy animals.

MATERIALS AND METHODS

Bovine sera

Sera from 29 animals, which were found at post-mortem to satisfy the criteria of BSE and 18 animals which did not, were supplied by the Central Veterinary Laboratory (CVL) (New Haw, Addlestone, Surrey), an executive agency of the U.K. Ministry of Agriculture, Fisheries and Food (MAFF). The 18 animals which did not have BSE had been referred to CVL because of abnormal behaviour but post-mortem examinations carried out by MAFF had excluded BSE.

Furthermore, 30 sera from animals aged less than 30 months (A<30M) (8 Friesians, 21 Hereford-Friesian and 1 Charolais-Friesian crossbreeds) and 28 sera from animals aged more than 30 months (A>30M) (all dairy Friesians), were used as further controls. These were collected

from a farm, kept under "organic farming" conditions where no case of BSE had been reported. Serum samples were obtained during routine herd testing.

Preparation of bacteria

Acinetobacter calcoaceticus was obtained from the National Collection of Industrial and Marine Bacteria Ltd. NCIMB 10694 (Aberdeen). Cultures were grown in 21 flasks on an orbital shaker for 2 days at 30°C, in 200 ml nutrient broth (Oxoid; 25 g/l). Flasks were inoculated with 10 ml of the corresponding starter culture left shaking at 37°C for 6 hours. Batch culture cells were harvested by centrifugation at 6000 r.p.m. for 20 minutes at 4°C (MSE 18,6 x 250 ml rotor). The pellets of cells were then washed three times with 0.15 M phosphate-buffered saline (PBS; pH 7.4) before being finally resuspended in 20 ml of PBS. A stock solution of the suspension was prepared by diluting in 0.05 M carbonate buffer (pH 9.6) to give an optical density (OD) reading of 0.25 on the spectrophotometer (Corning Model 258).

Enzyme-linked immunosorbent assay

ELISA assays were carried out in the conventional manner. Briefly ELISA plates were coated with bacteria overnight at 4°C and the non-specific sites blocked with PBS containing 0.1% Tween, 0.2% ovalbumin (Sigma, Grade III), plates washed and a 1/200 dilution of test or control serum added. The plates were incubated at 37°C for 1 hour, washed and rabbit anti-cow immunoglobulin (IgG + IgA + IgM) (1:4000) (Dako Ltd.) added. The plates were reincubated for 2 hours, washed and substrate added. The reaction was stopped with a 2 mg/ml solution of sodium fluoride (Sigma). The plates were read at 630 nm on a microtitre plate reader (Dynatech MR 600) and results expressed as OD ± S.E. All studies were carried out under code in that the tester did not know which were test or control sera. The mean OD units of total immunoglobulin antibodies in different groups were compared using Student's t-test.

ELISA METHOD SHEET

1. Dilute antigen in coating buffer, add 200μl to each well. Incubate overnight at 4°C wrapped in foil.
2. Wash out the antigen, using washing/incubation buffer; the wells of the tray should be completely full during the washing stages as the Tween-20 prevents any further protein from being absorbed onto the plastic. Wash 3 times, leaving for approx. 4 minute intervals at room temperature.
3. Incubate the plate at 37°C for 1hr with 0.2% Ovalbumin in washing/incubation buffer.
4. Add 200μl of test serum. Dilutions are made in washing/incubation buffer. Incubate for 2 hours at 37°C wrapped in foil.
5. Repeat washing process as in 2.
6. Add 200μl Horseradish peroxide HRP-conjugated second antibody, also diluted in washing/incubation buffer.
7. Repeat washing process as in 2.
8. Add 200μl substrate (ABTS) to wells; leave to develop colour for approx. 20 minutes in the dark at room temperature. Stop reaction with 100μl of stopping solution and read plate at 630nm.

RESULTS

Antibodies to A. calcoaceticus of total immunoglobulin (IgG + IgA + IgM) were significantly elevated in the BSE sera (mean ± SE: 0.99 ± 0.05) when compared to CVL controls (0.65 ± 0.06) ($t = 4.48$, $p<0.001$), organic farming controls aged more than 30 months (0.57 ± 0.03) ($t = 7.19$, $p<0.001$) and organic farming controls aged less than 30 months (0.53 ± 0.02) ($t = 8.64$, $p<0.001$). These results are shown in the attached Figure.

Legend to figure:

Antibody titres (bar = mean) for 30 controls aged less than 30 months (A<30m), 28 controls aged more than 30 months (A>30m), 18 controls from the Central Veterinary Laboratory (CVL) compared to 29 BSE sera, when tested against Acinetobacter calcoaceticus (Figure la) and E.coli (Figure lb). (Dashed line represents 95% confidence limits for mean of controls: A<30m + A>30m - one tailed test) (OD = optical density).

There was no significant difference between the CVL controls and the organic farming controls aged more than 30 months, but there was a small, statistically significant difference with the sera from animals aged less than 30 months ($t = 2.41$, $p < 0.05$). A re-examination of the CSL control serum with the highest anti-Acinetobacter level of 1.16 OD, showed that it came from a clinically normal control animal, diagnosed as negative to BSE on the statutory diagnostic criteria, and it was also negative when tested for scrapie associated fibrils. This case did however have white matter vacuolation of the substantia nigra and internal capsule, although this had been seen before and not considered significant.

One clear result from these studies, is that in at least in one "transmissible spongiform encephalopathy" (TSE), namely BSE, a specific immune response can be demonstrated against a microbe that is found readily in the environment of cattle and which also happens to possess a molecular sequence resembling bovine myelin.

Other forms of spongiform encephalopathy including Creuzfeld Jacob disease (CJD) and Multiple Sclerosis (MS) are open to explanation on the same model as indicated for BSE. CJD sera and MS sera are currently under test to confirm the presence of cross-reacting antibodies.

CLAIMS

1. A diagnostic test for spongiform encephalopathy and other demyelinating conditions in mammals which comprises assaying antibodies present in the mammal which bind to an antigenic peptide which exhibits molecular mimicry of a mammalian myelin peptide.

2. A test according to Claim 1, in which the mammalian myelin peptide has the sequence FSWGAEGQK.

3. A test according to Claim 1 or 2, for BSE in cattle.

4. A test according to Claim 3, using as the test antigen whole bacteria of an Acinetobacter, Agrobacterium, or Ruminococcus species.

5. A test according to Claim 4, using bacteria of the species Acinetobacter calcoaceticus, Agrobacterium tumefaciens, or Ruminococcus albus.

6. A test according to Claim 3, using as the test antigen a peptide derived from bacteria specified in Claim 4 or 5.

7. A test according to Claim 6, using a peptide of sequence ISRFAWGEV, YTRFTWGAP, or YTQFEISAE.

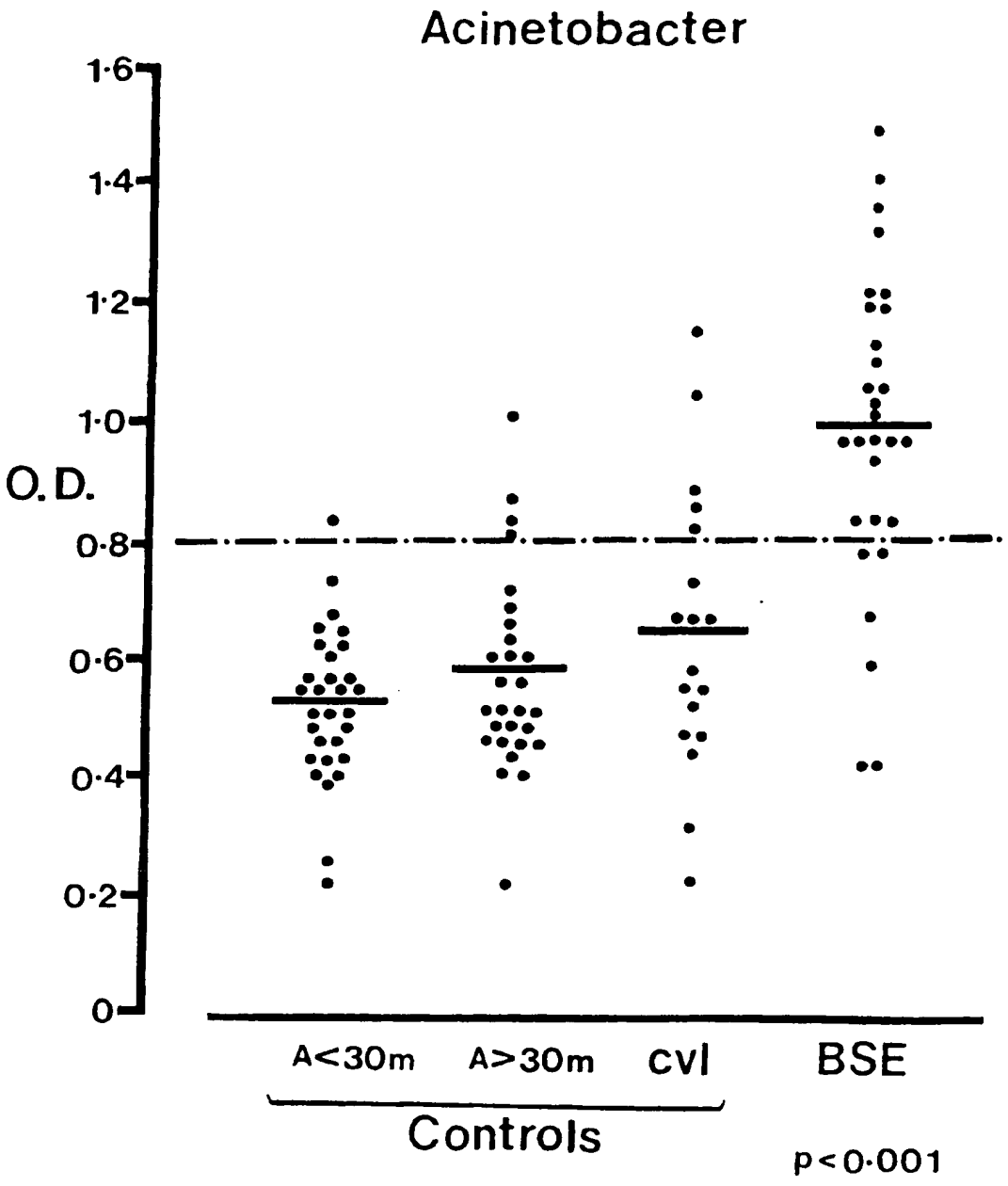
8. A test according to Claim 6 or 7, in which the peptide used is a synthetic peptide.

9. A method of testing for BSE in cattle which comprises assaying sera collected from the cattle for antibodies to a species of Acinetobacter, Agrobacterium or Ruminococcus, or a peptide having a sequence present in said species which mimics a peptide of bovine myelin and identifying animals having a level of antibodies at least about two standard deviations above that of healthy control animals.

10. A method according to claim 9, in which the bovine myelin peptide has the sequence FSWGAEGQK.

11. A diagnostic test kit for BSE in cattle comprising as test antigen a species of Acinetobacter, Agrobacterium or Ruminococcus, or a peptide having a sequence present in said species which mimics a peptide of bovine myelin.

12. A test kit according to claim 11, in which the test antigen is a peptide which mimics the sequence FSWGAEGQK.


Acinetobacter
1·6
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O.D.
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A<30m
A>30m
cvl
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Controls
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INTERNATIONAL SEARCH REPORT

Interr ·al Application No
PCT/GB 97/02667

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 80, no. 11, 18 March 1974 Columbus, Ohio, US; abstract no. 56313, A. WAJGT.: "Assessment by immunofluorescence methods of humoral antimyelin antibody in rats with cyanide encephalopathy." page 68; column 1; XP002052988 see abstract & ANN. IMMUNOL. (POZNAN), vol. 5, no. 1-2, 1973, pages 51-58, --- -/--	1

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex

" Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
22 January 1998	04/02/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 97/02667

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	B. H. TOH ET AL.: "The 200- and 150-kDa neurofilament proteins react with IgG autoantibodies from patients with kuru, Creutzfeldt-Jakob disease, and other neurologic diseases." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 82, May 1985, WASHINGTON US, pages 3485-3489, XP002052986 ---	
A	R. L. SIDMAN ET AL.: "Transmissible spongiform encephalopathy in the gray tremor mutant mouse." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 82, January 1985, WASHINGTON US, pages 253-257, XP002052987 ---	
A	CHEMICAL ABSTRACTS, vol. 109, no. 21, 21 November 1988 Columbus, Ohio, US; abstract no. 187890, M. P. MCKINLEY ET AL.: "Developmental regulation of prion protein mRNA in brain." page 484; column 2; XP002052989 see abstract & CIBA FOUND. SYMP., vol. 135(Novel Infect. Agents Cent. Nerv. Syst.), 1988, pages 101-116. -----	

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11 November 1999 (11.11.99)

(54) Title: DIAGNOSIS OF SPONGIFORM OR DE-MYELINATING DISEASE

(57) Abstract

A method for detecting a de-myelinating disease or spongiform encephalopathy in mammals comprises testing a biological sample obtained from the mammal for IgA antibodies indicative of infection by an *Acinetobacter* species. The *Acinetobacter* species is one which presents to the mammal an antigen which exhibits molecular mimicry with the myelin of the mammal e.g. *Acinetobacter calcoaceticus*. The antibodies tested for are antibodies which bind to an epitope present in or derived from the *Acinetobacter* species or to a prepared peptide sequence corresponding thereto or to a conformationally similar peptide sequence e.g. the peptide sequence RFSAWGAE or ISRFAWGEV. The method tests for bovine spongiform encephalopathy, multiple sclerosis and Creutzfeldt-Jacob disease in humans. A test kit uses as the test antigen the whole *Acinetobacter* organism or at least one prepared peptide sequence as described above and a secondary antibody against the human, bovine, or other mammalian IgA.

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DIAGNOSIS OF SPONGIFORM OR DE-MYELINATING DISEASE

This invention relates to the diagnosis of de-myelinating diseases and spongiform encephalopathies in animals and humans.

In our copending application WO 98/13694 we have disclosed a new diagnostic test for spongiform encephalopathies and other de-myelinating conditions in mammals. The test disclosed in our prior application is based on a model of the genesis of this pathological state which is applicable to the various forms in which it is manifest in humans and animals. In relation to the bovine spongiform disease this model provides an alternative to the current theory based on the formation of prions. Briefly, this new model is based on the phenomenon of molecular mimicry according to which mammals exposed to certain bacteria having peptide sequences which mimic myelin peptides experience an auto-immune reaction. In our prior application we indicated that human de-myelinating diseases were also open to the same explanation according to our new model disclosed therein.

According to the present invention, a method for detecting a de-myelinating disease or spongiform encephalopathy in mammals comprises testing a biological sample obtained from the mammal for IgA antibodies indicative of infection by an *Acinetobacter* species. We believe that infective micro-organisms of these species present to the mammal an antigen which exhibits molecular mimicry with the myelin of the mammal. The phenomenon of molecular mimicry has been explained in our above-mentioned prior

application WO 98/13694, the contents of which are hereby incorporated by reference.

We have now confirmed the presence of elevated levels of certain antibodies in human sera of patients suffering from multiple sclerosis (MS). These are the IgA antibodies to *Acinetobacter* species e.g. *Acinetobacter calcoaceticus*, the same organisms for which antibodies were previously found in BSE sera. Similar results have been obtained for Creutzfeldt-Jakob disease (CJD). Tests for antibodies in sera from patients who had died of CJD also show increased levels, this being especially marked for the IgA antibody sub-class. The same IgA specificity also applies to bovine sera used for the tests described in our above-mentioned copending application.

It is clear that humans suffering from MS and CJD and cows suffering from BSE all have very significantly raised levels of *Acinetobacter calcoaceticus* IgA antibodies in their blood. Tests for such antibodies in sera from living subjects at an early stage make it possible to identify those liable to develop these diseases. The present invention opens up the opportunity of early treatment of these infections e.g. by use of an appropriate antibiotic to prevent further auto-immune attack on the subjects' own myelin.

As also indicated in our application WO 98/13694, *Acinetobacter calcoaceticus* is one species of *Acinetobacter* which provides an antigen which stimulates the formation of antibodies which cross-react with the mammalian myelin . Antibodies have been demonstrated to react with several strains of this species including 17905, AC606, SP13TV, 105/85, and 11171. These strains are in the

Reference Centre for Acinetobacter species held by Dr Kevin Towner, Public Health Laboratory, University of Nottingham, U.K.

In carrying out the present invention, the test is for antibodies which bind to an epitope present in or derived from the *Acinetobacter* species. The antigen used in the test may be the whole organism or at least one prepared peptide sequence corresponding to an *Acinetobacter* epitope. Alternatively, peptide sequences may be used which have minor variations in amino-acid sequence from the above-mentioned epitopes or prepared peptides but are conformationally sufficiently similar to them that they also bind to the relevant antibodies. For example, peptides having the sequence RFSAWGAE or ISRFAWGEV may be used.

A test kit for use according to the invention therefore contains at least one test antigen as just indicated. In order to reveal IgA antibodies the kit also contains a secondary antibody against the human, bovine, or other mammalian IgA.

As indicated in WO 98/13694, antibodies are assayed and a positive result is indicated by levels of antibodies at least about two standard deviations above that of control samples.

In view of the greater specificity of the IgA antibodies in the immune response it may be concluded that the mechanism of infection with *Acinetobacter* is via the mucous membranes of the body, the primary sites being the gut or the nasal passages. Since a further correlation has been observed between MS sufferers and patients with major sinus infections, it is probable that the nasal passages

EXAMPLE

The assay for the above mentioned organisms is described in our co-pending application mentioned above. The improved method used herein is as follows:-

ELISA TEST

1) Aliquots of 200 ul of the diluted suspension of *Acinetobacter calcoaceticus* (NCIMB 10694, Aberdeen) grown in nutrient broth are absorbed onto 96 well flat bottomed rigid polystyrene microtitre plates overnight at 4°C.

2) The plates are then washed 3 times with phosphate buffered saline (PBS), 0.1% (v/v) Tween 20.

3) Aliquots of 200 µl of blocking solution (0.2% w/v ovalbumin, 0.1% v/v Tween 200 in PBS is added to each well and incubated for one hour at 37°C.

4) The plates are then washed 3 times with PBS.Tween 20.

5) Aliquots of 200 µl serum samples (test or control) diluted 1/200 in PBS. Tween 20 is added and incubated for 2 hours at 37°C.

6. The plates are then washed 3 times with PBS.Tween 20.

7) Aliquots of 200 µl of peroxidase conjugated rabbit anti-human IgA or rabbit anti-cow Iga , diluted 1/4000 (cow) (or 1/500 for human) with PBS.Tween 20 are added and incubated for 2 hours at 37°C.

8) The plates are then washed 3 times with PBS.Tween 20.

9) The development of the colorimetric assay takes place at room temperature for 20 minutes, after the addition of 200 µl per well of 0.5 mg/ml (2,2'-azinobis(3-ethylbenz-thiazoline-6-sulphonic acid) in citrate/phosphate buffer, pH 4.1, containing 0.98 mM hydrogen peroxide.

10) the reaction is then stopped with 100 µl of 2 mg/ml sodium fluoride and optical densities measured at a wavelength of 630 nm with a micro-ELISA plate reader.

Results for MS and CJD are shown in the attached Figure 1 and those for BSE are shown in Figure 2. These give the titres of IGA *Acinetobacter* antibodies in MS and CJD sera, BSE sera, and control sera. The dashed line represents the 95% confidence limits of the controls.

CLAIMS

1. A method for detecting a de-myelinating disease or spongiform encephalopathy in mammals which comprises testing a biological sample obtained from the mammal for IgA antibodies indicative of infection by an *Acinetobacter* species.

2. A method according to claim 1, in which the *Acinetobacter* species is one which presents to the mammal an antigen which exhibits molecular mimicry with the myelin of the mammal.

3. A method according to claim 1 or 2, in which the antibodies are indicative of prior infection by *Acinetobacter calcoaceticus*.

4. A method according to claim 1, 2, or 3, in which the antibodies tested for are antibodies which bind to an epitope present in or derived from the *Acinetobacter* species or to a prepared peptide sequence corresponding thereto or to a conformationally similar peptide sequence.

5. A method according to claim 4, in which the epitope contains the peptide sequence RFSAWGAE.

6. A method according to claim 4, in which the epitope is the peptide sequence ISRFAWGEV.

7. A method according to any of claims 1 to 6, in which the disease tested for is bovine spongiform encephalopathy.

8. A method according to any of claims 1 to 6, in which the disease tested for is multiple sclerosis in humans.

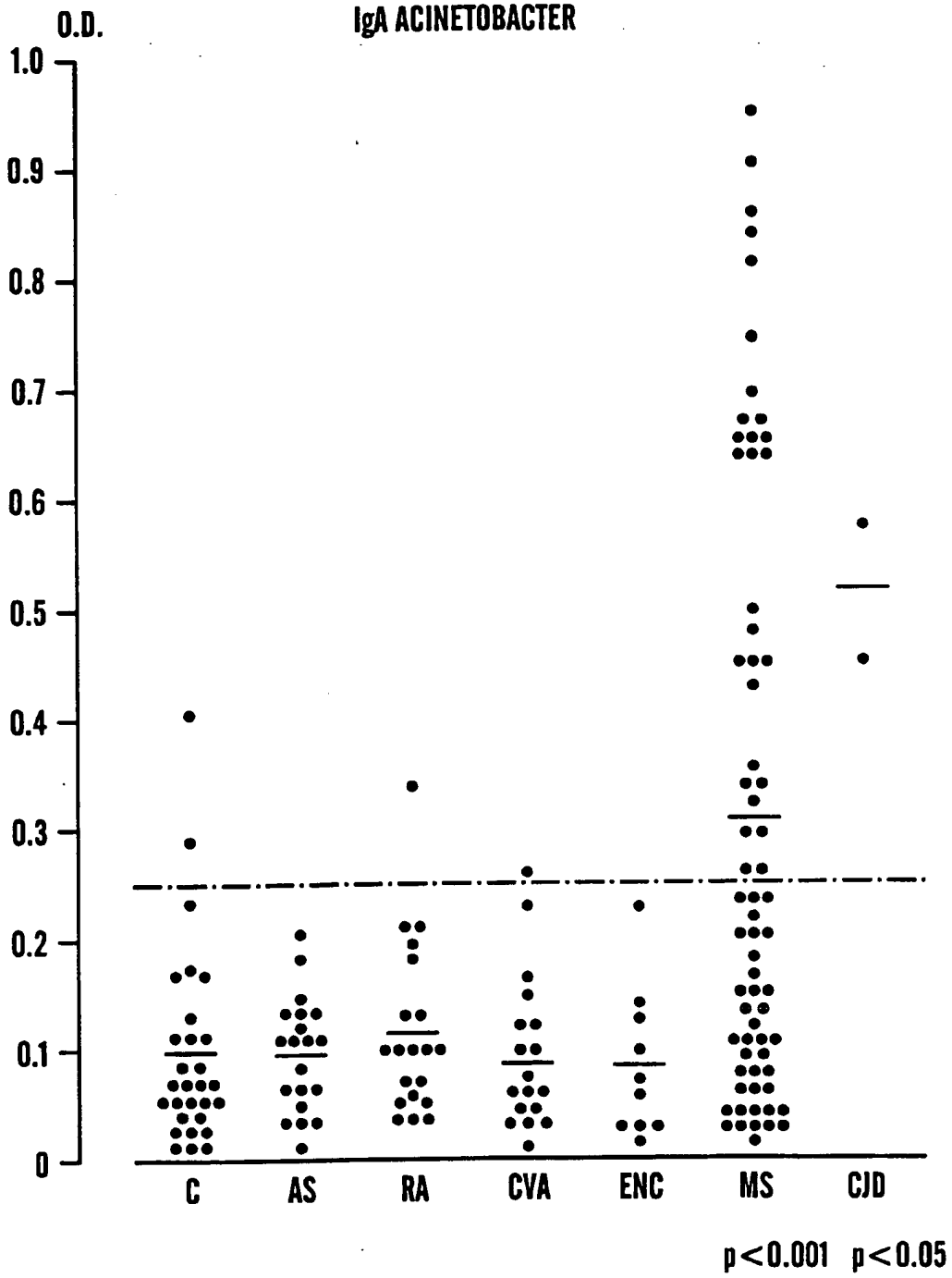
9. A method according to any of claims 1 to 6, in which the disease tested for is Creutzfeldt-Jacob disease in humans.

10. A method according to any of the preceding claims in which antibodies are assayed and a positive result is indicated by levels of antibodies at least about two standard deviations above that of control samples.

11. A test kit for use with a method according to any of the preceding claims, in which the test antigen is the whole *Acinetobacter* organism or at least one prepared peptide sequence corresponding to an *Acinetobacter* epitope or a variant peptide sequence which is conformationally sufficiently similar to it to bind to the relevant antibodies, and a secondary antibody against the human, bovine, or other mammalian IgA.

12. A test kit according to claim 11, comprising a peptide having the sequence RFSAWGAE or ISRFAWGEV.

13. A test kit according to claim 11 or 12, in which the secondary antibody is a rabbit anti-human IgA or rabbit anti-bovine IgA.



LEGEND: IgA ANTIBODIES TO ACINETOBACTER BACTERIA, MEASURED BY ELISA IN HEALTHY CONTROLS (C) AND PATIENTS WITH ANKYLOSING SPONDYLITIS (AS), RHEUMATOID ARTHRITIS (RA), CEREBRO-VASCULAR ACCIDENTS (CVA), VIRAL ENCEPHALITIS (ENC), MULTIPLE SCLEROSIS (MS) AND CREUTZFELDT-JAKOB DISEASE (CJD). (p-VALUES INDICATE SIGNIFICANCE COMPARED TO CONTROLS)

Fig. 1

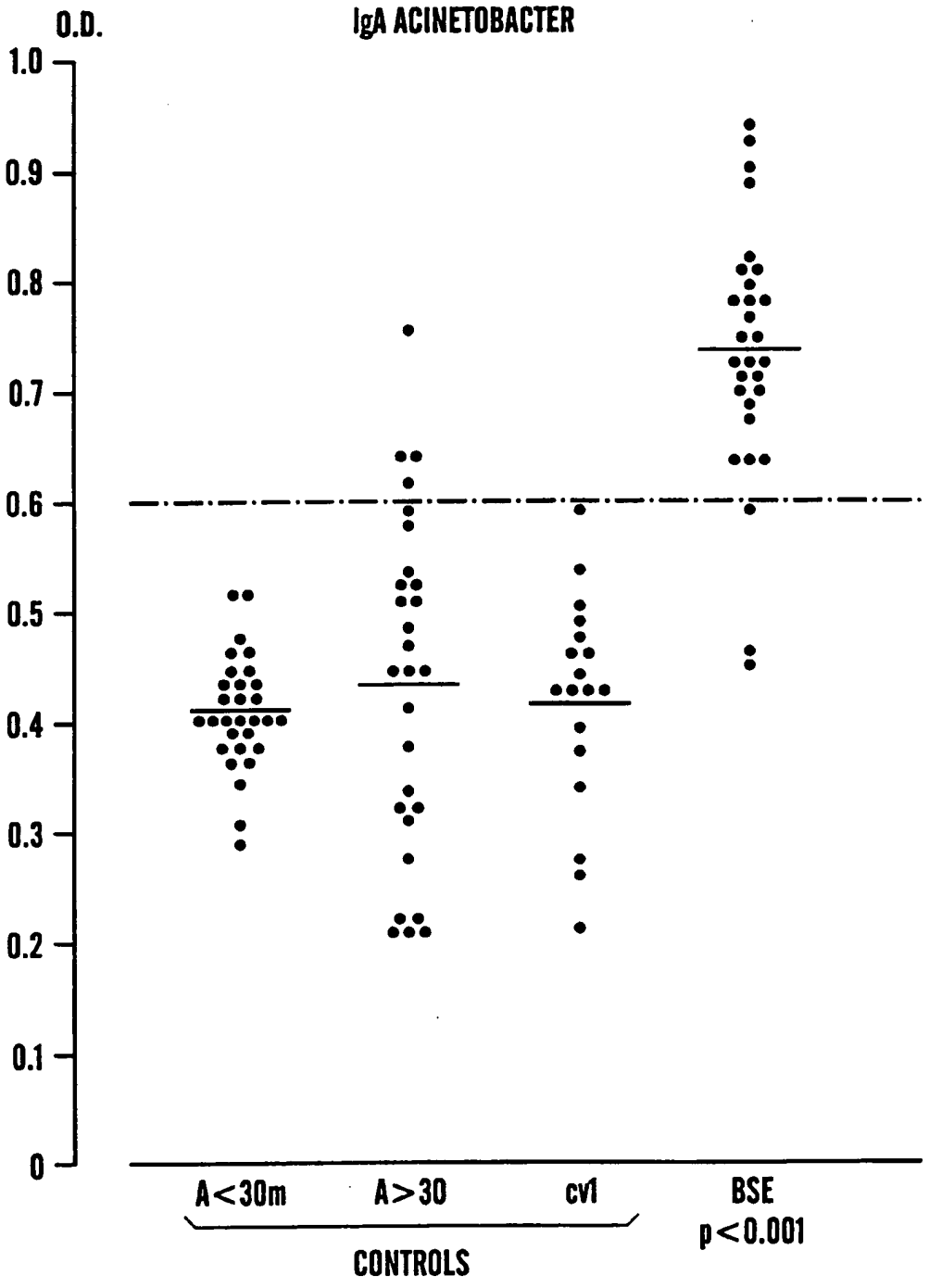


Fig.2

INTERNATIONAL SEARCH REPORT

Intern · ıal Application No
PCT/GB 99/00876

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 80, no. 11, 18 March 1974 (1974-03-18) Columbus, Ohio, US; abstract no. 56313, A. WAJGT.: "Assessment by immunofluorescence methods of humoral antimyelin antibody in rats with cyanide encephalopathy." page 68; column 1; XP002052988 abstract & ANN. IMMUNOL. (POZNAN), vol. 5, no. 1-2, 1973, pages 51-58, --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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22 September 1999	30/09/1999

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	B. H. TOH ET AL.: "The 200- and 150-kDa neurofilament proteins react with IgG autoantibodies from patients with kuru, Creutzfeldt-Jakob disease, and other neurologic diseases." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 82, May 1985 (1985-05), pages 3485-3489, XP002052986 WASHINGTON US ---	
A	R. L. SIDMAN ET AL.: "Transmissible spongiform encephalopathy in the gray tremor mutant mouse." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 82, January 1985 (1985-01), pages 253-257, XP002052987 WASHINGTON US ---	
A	CHEMICAL ABSTRACTS, vol. 109, no. 21, 21 November 1988 (1988-11-21) Columbus, Ohio, US; abstract no. 187890, M. P. MCKINLEY ET AL.: "Developmental regulation of prion protein mRNA in brain." page 484; column 2; XP002052989 abstract & CIBA FOUND. SYMP., vol. 135(Novel Infect. Agents Cent. Nerv. Syst.), 1988, pages 101-116, ---	
P,X	WO 98 13694 A (KING'S COLLEGE) 2 April 1998 (1998-04-02) the whole document -----	1-13

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INTERNATIONAL SEARCH REPORT

ormation on patent family members

Intern··nal Application No
PCT/GB 99/00876

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9813694 A	02-04-1998	EP 0929813 A	21-07-1999

International Preliminary Examining Authority
European Patent Office
Erhardtstrasse 27
D-80331 Munich
Germany

Our Ref : IT/SC/N8976

18 October 2000

Dear Sirs,

International Patent Application PCT/GB99/03936

We now respond to the Written Opinion dated 8 August 2000 on the above application.

First, we wish to replace the expression "myelin neurofilaments" throughout this application by the more appropriate term "neurofilaments". As is well known, neurofilaments are contained within a protective myelin sheath, and the original expression was used in this sense. However, it is evident that the expression has caused an apparent misunderstanding, in being taken to mean myelin "fragments" e.g. as in the Examiner's reference to "fragments" of myelin protein in D3.

We therefore file the enclosed revised version of the application with the correct term "neurofilaments" used throughout in place of "myelin neurofilaments". Apart from the consequential change on new page 3 line 17 (removal of "thereof") this is the only change that has been made. Clerical errors on original page 5, lines 6 and 10, and on original page 6 line 11 have also been corrected.

The Examiner will note that the correct term "bovine neurofilaments" has been given in section (1) of the ELISA test described on original page 3 line 24 of the application in relation to Antigen B obtained from Sigma Chemical Co.

A copy of the relevant page of Sigma catalogue entry for their product is enclosed in support of our proposal. This refers to both neurofilaments and antibodies to neurofilaments. Also, on page 5 of the application (line 5 from the foot of the page) the two antigens are correctly referred to and again on page 6 of the application the correct term has been given in relation to the MAN index.

Should the Examiner see any difficulty over this change of terminology we would appreciate the opportunity to discuss the matter by telephone.

Referring now to the relevant numbered paragraphs in the Written Opinion we have the following comments:

1.2 D3 refers to myelin protein or fragments in the context of therapy but not diagnosis. It does not refer to neurofilaments. D3 does not deprive claim 8 of novelty. We believe that a claim to a kit comprising either of these agents as test antigen for the specified diseases is justifiable.

2.1 The Examiner refers to D1 and D2. D1 is a scientific literature counterpart to D2 and both come from the same source (the present Applicant). These disclosures are based on the postulate that the bovine disease is caused by infection with Acinetobacter species.

But in neither of D1 and D2 is there any suggestion or hint of the possibility of a much more convenient diagnostic test using the available materials (myelin or neurofilaments) as defined in claim 1. It was not within the foresight of the present inventor to propose the claimed method even though he is the champion of the theory of a molecular mimicry mechanism underlying these diseases.

Furthermore, the Examiner seems to have overlooked the fact that in the first paragraph of page 6 of the application the MAN test is said to consist of separate measurements of myelin and neurofilaments "as well as to specific antibodies" to the Acinetobacter species. The MAN test therefore indicates that the detected myelin and neurofilament antibodies are not the same antibodies as those which are detected as specific to the Acinetobacter organisms. The Examiner's conclusion on this point is therefore not correct.

It is not understood why the Examiner refers to the acinetobacter species at all in the argument against claim 1 and its dependent claims. Testing for these separate antibodies in combination is not introduced until claims 7 and 10 of the application. This is the preferred method and test kit for achieving maximum certainty of diagnosis in the context of the molecular mimicry hypothesis which lies at the root of the present inventor's whole approach to this problem.

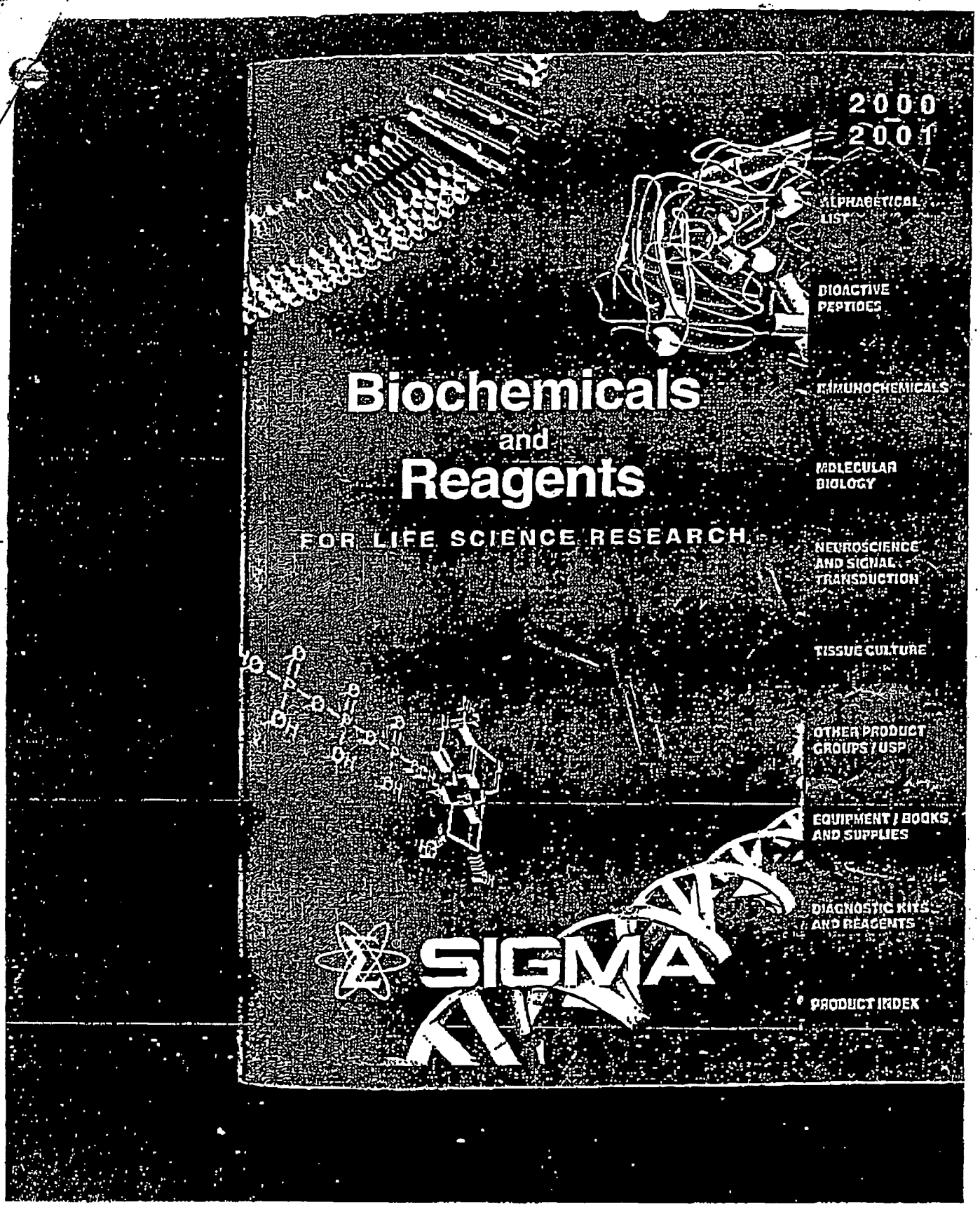
Item VII

As pointed out above D1 is the literature paper corresponding to D2 which is mentioned in the present application. In our view, D3 is not of sufficient relevance to justify mention in the present context.

Accordingly, it is not seen necessary to make any significant changes to the claims of the present application, or to amend the description other than as indicated at the start of this response.

Yours faithfully,

Ian Tollett
encs.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Reagents
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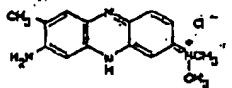
ALPHABETICAL LIST OF COMPOUNDS

PRODUCT NUMBER			£
	(Continuation of) NEURAMINIDASE		
N 5631	Type VIII: Chromatographically purified From Clostridium perfringens Prepared from Type V Dialyzed and lyophilized powder containing approx. 90% protein (Biuret) Activity: 10-20 units per mg protein (NAN-lactose) and approx. 4 units per mg protein (mucin). May contain proteases and NAN-aldolase. (900)-676	1 unit 5 units 10 units 50 units	37.10 122.00 200.40 715.20
N 2133	Type X: From Clostridium perfringens A further purification by affinity chromatography of our Type VIII [N 5631]. Dialyzed and lyophilized powder containing approx. 85% protein [Bradford]. Activity: 150-400 units per mg protein (NAN-lactose). (900)-676	1 unit 5 units 10 units 50 units	43.90 145.30 238.70 852.20
N 6021	Type II-A: Insoluble enzyme attached to beaded agarose. From Vibrio cholerae Lyophilized powder stabilized with lactose. Activity: 45-135 units per g of agarose (NAN-lactose). One ml of gel will yield 1.5-4.5 units. Prepared from Neuraminidase, Type II. R: 20-42/43-36/37/38 S: 26-36-22	1 unit 5 units	81.10 320.00
N 5254	Type VI-A: Insoluble enzyme attached to beaded agarose. From Clostridium perfringens Suspension in 2.0 M $(NH_4)_2SO_4$ solution, pH 7.0. Activity: 0.6-1.8 units per ml of gel (NAN-lactose). One gram of agarose will yield 20-60 units. Prepared from Neuraminidase, Type VI.	1 unit 10 units	48.80 289.10
N 4883	Type X-A: Insoluble enzyme attached to beaded agarose. From Clostridium perfringens Suspension in 2.0 M $(NH_4)_2SO_4$ solution, pH 7.0. Activity: 20-30 units per gram of agarose (NAN-lactose). One ml gel will yield 0.6-1.0 unit. Prepared from Neuraminidase, Type X.	1 unit 5 units	93.20 326.60
	NEURAMINIDASE, Positionally Specific Recombinant; expressed in E. coli Solution in 20 mM Tris-HCl, pH 7.5, 25 mM NaCl Unit Definition: One unit will hydrolyze 1 μmole of 4-methylumbelliferyl α-N-acetylneuraminide per min at pH 5.0 at 37°C. Absence of contaminants: enzymes are expressed in glycosidase-free hosts; contaminating β-galactosidase, α-mannosidase, β-hexosaminidase, α-fucosidase, and proteases are not detectable. Provided with 5× reaction buffer (250 mM sodium phosphate, pH 6.0). (900)-676		
N 7271	α-2→3-Neuraminidase Releases α-2→3-linked N-acetylneuraminic acid from complex oligosaccharides.	0.2 unit	171.20
N 5521	α-2→(3,6)-Neuraminidase Releases α-2→3- and α-2→6-linked N-acetylneuraminic acid from complex oligosaccharides.	0.4 unit	171.20
N 8271	α-2→(3,6,8,9)-Neuraminidase Releases α-2→3-, α-2→6-, α-2→8, and α-2→9-linked N-acetylneuraminic acid from complex oligosaccharides.	0.2 unit	171.20
	NEURAMIN-LACTOSE See: N-Acetylneuramin-lactose Page 41		
	NEUROACTIVE COMPOUNDS, NEUROCHEMICALS, AND RELATED COMPOUNDS See: Neurochemicals Section Page 1636		
	NEUROFILAMENT, ANTIBODIES TO See: Immunochemicals Page 1130		
	NEUROFILAMENTS	Protein	
N 1022	From Bovine Spinal Cord Lyophilized from a solution containing 6 M urea, 10 mM sodium phosphate, 5 mM EDTA and 1% β-mercaptoethanol, pH 7.5. Intermediate filaments found in axons of large myelinated fibers, most neurons, astrocytes and Schwann cells Prepared using a modification of Dahl, D., et al., Anal. Biochem., 128, 165 (1982).	500 μg	349.50
	NEUROGRANIN FRAGMENT 28-43 See: Bioactive Peptides Page 1069		
	NEUROKININS See: Bioactive Peptides Page 1078		
	NEUROMEDINS See: Bioactive Peptides Page 1078		
	NEUROPEPTIDE K See: Bioactive Peptides Page 1054		
	NEUROPEPTIDE Y See: Bioactive Peptides Page 1054		
	NEUROPHYSIN I	100 μg	120.10
N 2404	From Bovine Pituitaries A protein found in vasopressin- and oxytocin-containing neurons in the hypothalamus that is associated with the transport of these hormones to the posterior pituitary (63231-69-4)		
	NEUROTENSIN and RELATED PEPTIDES See: Bioactive Peptides Page 1061		
	NEUROTOXINS See: Toxins, Snake Page 964		
	NEUROTOXINS, Kits of See: Venoms Page 1011		
	NEUROTRANSMITTERS, NEUROPEPTIDES, NEURONAL ENZYMES AND HORMONES, ANTIBODIES TO See: Immunochemicals Page 1196		
	NEUTRALIZED CHARCOAL See: Charcoal, Activated Page 231		
	NEUTRAL RED (C.I. 50040; 3-Amino-7-dimethylamino-2-methylphenazine hydrochloride) pH range: 6.8 (red) - 8.0 (yellow). Useful as an indicator for preparing neutral red paper, and as a biological stain.  [553-24-2] $C_{15}H_{17}N_4 \cdot HCl$ FW 288.8		
N 7005	Purified Dye Content: >90% See also: Tissue Culture Media and Reagents Page 1762	1 g 5 g 25 g	13.80 40.70 132.60
N 8906	Dye Content: ≥80%	1 g 5 g 25 g	10.20 33.90 112.90
N 2880	Practical Grade Dye Content: Approx. 60%	25 g 100 g	22.40 65.40

(Continued)

6. In my opinion, after reading the text of the application as a whole, I believe that the term has obviously been used by mistake, in place of the correct term "neurofilaments", and that persons skilled in the field of Neuroimmunology would reach the same conclusion as I have. This conclusion is supported by the fact that, in the Experimental section of the application, reference has been made to commercially available neurofilaments from bovine spinal cord as one of the test antigens used in the method and kit claimed in the application. These are stated in the manufacturer Sigma's catalogue to be "intermediate filaments found in axons of large myelinated fibers, most axons, astrocytes and Schwann cells". I understand that the Applicant has previously drawn attention to this fact.

DECLARED by me ----------

KENNETH J SMITH

Date 4 December 2003

In the matter of International Patent Application
WO 00/31545, Kings College, London

DECLARATION

I, Kenneth J Smith, do hereby declare and state :-

1. I am Professor of Neurobiology and Head of the Neuroinflammation Research Group in the Department of Neuroimmunology, Guy's, King's, and St. Thomas' School of Medicine, Guy's Campus, Kings College London.

2. I have been asked to advise on the meaning of a term used in International Patent Application WO 00/31545 as published on 2nd June 2000. I have not considered any other aspect of this application.

3. The term in question is "myelin neurofilaments". This term has no recognised usage in the field of Neuroscience. Myelin is the insulating layer that surrounds the axons of many neurons in the central and peripheral nervous system. Neurofilaments are structures present in neurons. Myelin and neurofilaments are separate and distinct from one another. Antibodies to myelin proteins and antibodies to neurofilaments are found in certain disease situations but these are separate and distinct antibodies.

4. I have considered the possibility that the term "myelin neurofilaments" is being used in this application to denote some particular type of neurofilaments, in which case I would have expected to find some explanation of its intended meaning. No such explanation is to be found in the description or the claims of the application. I therefore believe that no special use of the term is being made.

5. I have considered the possibility that the term "myelin neurofilaments" has been used by the patent draftsman by mistake, in place of the sole term "neurofilaments" having the established meaning given in paragraph 3 above. I understand that the Applicant has affirmed this to be so.